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Orexins and Their Receptors in the Human Retina

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Key Words
Human retina · Hypocretin · Ocular vessels · Orexins · Receptors · Retina

Abstract
Objectives: Orexins A and B are neuropeptides involved in the regulation of feeding behavior, energy homeostasis and arousal. In the human retina, however, immunohistochemical localization of orexins and their receptors, OX-R1 and OX-R2, has not been ascertained. ***Methods:*** We localized orexins A and B, OX-R1 and OX-R2 in the human retina using immunohistochemistry. Retinae from 2 Alzheimer's disease (AD) patients provided preliminary evidence for possible orexin alterations. ***Results:*** Orexin A, orexin B and OX-R1 were localized in ganglion and amacrine cells, cellular processes in the inner and outer plexiform layer and in the inner segments of photoreceptor cells. There was no OX-R2 immunoreactivity in the retina. The staining intensity for both orexins was decreased in the AD patients. ***Conclusion:*** This immunohistochemical study provides the first evidence for the distribution of orexin A, orexin B and OX-R1 in the human retina. The localization pattern suggests a modulatory role for orexins in the interactions of those retinal cells which transmit light information to the suprachiasmatic nuclei, and thus may be involved in circadian rhythm entrainment.

Introduction

The orexins/hypocretins represent a novel family of neuropeptides which were independently identified by two groups [1, 2]. De Lecea et al. [1] described a hypothalamus-specific mRNA that encodes preprohypocretin, which was thought to be the precursor of two neuropeptides, namely hypocretin-1 and -2. At about the same time, Sakurai et al. [2] identified two neuropeptides that activate two closely related orphan G-protein-coupled receptors. These were named orexins after the Greek word for appetite, because these peptides stimulated food intake. The proteolytic cleavage of a precursor releases two peptides, orexin A and orexin B, which share 46% sequence identity [3]. It is now accepted that the terms orexin and hypocretin represent identical molecular entities [3].

Anatomical studies in rodents have determined the hypothalamus as the main localization of these peptides, where they are expressed in the lateral hypothalamic area, fornix and posterior hypothalamus [4–9]. In rodents, orexin-A- and -B-containing neurons project widely into the olfactory bulb, cerebral cortex, thalamus, and brainstem [4, 9].

There are less data dealing with the distribution of orexins in the human brain [10, 11]. The highest concentration of orexin A has been found in the human hypothalamus, followed by the thalamus, medulla oblongata, and pons [10]. Orexin-containing neurons localized to the

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perifornical region of the human posterior hypothalamus innervate all areas of the cerebral cortex, hypothalamus, locus ceruleus, raphe nuclei, midline thalamus and forebrain [11]. In human peripheral tissues, orexin A expression has been detected in ganglion cells of the thoracic sympathetic trunk and myenteric plexus, endocrine cells of the gastrointestinal tract and islet cells of the pancreas [12]. The unique neuronal distribution and innervation pattern of orexin neurons suggest complex roles of these peptides in autonomic and neuroendocrine control, including the regulation of feeding behavior and energy homeostasis [9], as well as arousal [11].

Orexins are considered to exert their functions through two specific orexin receptors, OX-R1 and OX-R2, both having a seven transmembrane domain topology, generated from two separate genes [2]. Orexin A has a high affinity to both receptors, whereas orexin B shows a 10-fold higher affinity to OX-R2 than to OX-R1. The distribution of both receptors has been determined in rats [13–18], where high levels of OX-R1 mRNA were detected in the ventromedial hypothalamic nucleus, thalamic nuclei, cerebral cortex, basal ganglia, tenia tecta, hippocampus, dorsal raphe and locus ceruleus [13, 15, 16]. OX-R2, on the other hand, was mainly expressed in the paraventricular nucleus, cerebral cortex, nucleus accumbens, subthalamic and paraventricular thalamic nuclei, and anterior pretectal nucleus [16, 17]. These findings support the hypothesis that orexins may have important actions not only on hypothalamic neurons controlling food intake and fluid balance, but also on different neuronal systems. Orexin receptor distribution has not yet been investigated in the human brain.

Orexins are directly involved in the regulation of sleep. Narcolepsy, a neurological disorder characterized by hypersomnia and catalepsy, is associated with orexin loss or rarely with orexin receptor gene mutations [19–21]. Orexins may be implicated in the circadian regulation of sleep propensity, given the presence of OX-R1 in the suprachiasmatic hypothalamic nuclei (SCN) [15]. Light information transduced to the SCN via the retina is necessary to entrain circadian rhythms [22–24], and the retina itself contains a circadian pacemaker [25]. Therefore, we investigated the distribution of orexin A, orexin B and their receptors in the retina using immunohistochemistry. Retina samples of 2 patients with Alzheimer's disease (AD) were also investigated, since AD, a neurodegenerative disorder characterized by cognitive decline, is often accompanied by circadian rhythm disturbances and SCN degeneration [26].

Table 1. Data of controls and AD patients

No.	Age years	Gender	Postmortem delay	Cause of death
Controls				
1	85	m	31 h	heart failure
2	97	f	26 h	heart failure
3	91	f	24 h	heart failure
4	69	f	20 h	pancreatitis
5	76	f	9 h 40 min	heart failure
6	88	f	31 h	heart failure
7	76	f	9 h	pneumonia
8	92	m	24 h 32 min	heart failure
9	85	f	6 h 33 min	heart failure
AD patients				
1	83	m	15 h	heart failure
2	84	m	17 h 30 min	pneumonia

Patients and Methods

Human Tissue

Eyes of 9 elderly control subjects without neurological or psychiatric disease (7 women and 2 men, mean postmortem delay 23 h 57 min) and 2 male AD cases (mean postmortem delay 16 h 15 min) were investigated (table 1). The diagnosis of AD was made on clinical evaluation and confirmed by postmortem neuropathological examination. The cause of death was heart failure in most cases, pancreatitis in 1 case and pneumonia in 2 cases (table 1). Sample collection was according to ethics committee criteria and the Declaration of Helsinki in 1975. The paraffin-embedded tissues were fixed in 4% paraformaldehyde and cut sagittally in 4-μm-thick consecutive sections.

Immunohistochemistry

Immunohistochemical detection of all antigens was done by peroxidase staining using the peroxidase substrate 3-amino-9-ethylcarbazole. The method has been reported in detail previously [24, 27]. Adjacent sections were incubated with antisera or primary antibodies against orexin A (rabbit anti-orexin-A serum, Peninsula Laboratories, San Carlos, Calif., USA), orexin B (rabbit anti-orexin-B serum, Peninsula Laboratories), OX-R1 (rabbit polyclonal antibody, Chemicon International, Temecula, Calif., USA), or OX-R2 (rabbit polyclonal antibody, Chemicon International). According to the company, the antisera against orexin A and orexin B show a 100% cross-reactivity with orexin A and B, respectively. The affinity-purified antibody against OX-R1 recognizes a 16-amino-acid sequence near the C-terminus of human OX-R1 and the antibody against OX-R2 was developed against a 19-amino-acid peptide near the N-terminus of human OX-R2 [1, 2]. The optimum concentration for all antisera and antibodies was experimentally determined to be 1:200. To test the specificity of the antisera and the antibodies, control samples were stained simultaneously following the same procedure as the test samples but omitting the antisera or the primary antibodies. After the peroxidase reaction with ACE, the samples were counterstained with Mayer's hemalum

Table 2. Semiquantitative data tabulating the intensity of immunoreactivity of orexin A, orexin B, and orexin receptors OX-R1 and OX-R2 in the retina of controls and AD patients

No.	Orexin A GC, AC, PC, IOPL	Orexin B GC, AC, PC, IOPL	OX-R1 GC, AC, PC, IOPL	OX-R2 GC, AC, PC, IOPL
Controls				
1	+++, ++, +++, ++	+++, ++, ++, ++	+++, ++, +++, ++	–
2	++, +, +, +	++, +, ++, +	+++, ++, ++, ++	–
3	+, ++, ++, ++	++, ++, ++, +	+++, ++, +++, ++	–
4	++, +, ++, –	++, +, –, –	+++, ++, +++, ++	–
5	+++, +++, ++, ++	+++, ++, +++, ++	+++, ++, +++, ++	–
6	++, ++, ++, ++	++, +, –, –	++, ++, +, +	–
7	+++, ++, ++, ++	+++, ++, +++, ++	+++, ++, +++, ++	–
8	+++, ++, ++, ++	+++, ++, ++, ++	+++, ++, ++, ++	–
9	++, ++, ++, ++	++, ++, ++, +	+++, ++, ++, ++	–
AD patients				
1	++, +, +, +	++, +, +, +	++, +, ++,+	–
2	+, –, –, –	+, –, –, –	++, +, –, +	–

Staining intensity: – = No immunoreactivity; + = slight; ++ = moderate; +++ = high; GC = ganglion cells including nerve fiber layer; AC = amacrine cells; PC = photoreceptor cells ; IOPL = inner and outer plexiform layers.

to mark the cell nuclei. All sections were assessed for localization and intensity of specific immunoreactivities on a semiquantitative scale by two blinded observers.

Results

We observed orexin-A-, orexin-B- and OX-R1-immunoreactive structures in the human retina (table 2). No ocular vessels, including central, retinal and choroidal arteries and veins, stained for any of the investigated antigens.

Different cells and cellular processes within the retina revealed an intense immunoreaction for orexin A, orexin B and OX-R1 (fig. 1A–C, fig. 2A, B), whereas OX-R2 immunoreactivity was entirely missing (fig. 1D, table 2). Control adjacent sections omitting the primary antibody were not immunoreactive for any of the tested antigens, confirming the specificity of the immunoreaction (fig. 1E). Starting at the inner layers of the retina, the ganglion cells within the ganglion cell layer were the most prominent cellular structures which intensively stained for orexin A, orexin B or OX-R1 (fig. 1A–C). The immunoreaction accumulated in a perinuclear pattern in the ganglion cell somata and in the primary cellular processes. The ganglion cell processes within the nerve fiber layer were also positive for the observed antigens. Adjacent to the ganglion cell layer, cell processes within the inner plexiform layer were also positive for all three antigens (fig. 1A–C). The inner nuclear layer revealed single orexin-A-, orexin-B- or OX-R1-immunoreactive cells (fig. 1A–C). According to their cellular structure, these cells could be identified as amacrine cells. The outer plexiform layer also contains orexin-A-, orexin-B- or OX-R1-immunoreactive cell processes both in the central and mid-peripheral parts of the retina (fig. 1A–C). The inner segments of the photoreceptor cells, located adjacent to the outer nuclear layer containing the somata of the photoreceptor cells, stained for all three antigens (fig. 2A, B). Finally, the Henle layer, interposed between the inner and outer nuclear layer, also contained immunolabeling for all antigens except OX-R2 (fig. 1A–C). The 9 control cases in the present study showed individual differences in the staining intensity for the observed antigens (table 2). Interestingly, even those with the weakest staining for orexin A or orexin B still revealed very intense OX-R1 staining (table 2, case No. 2 and No. 4).

In the 2 AD cases, the retina displayed similar cellular distribution patterns for orexin A (fig. 1F), orexin B or OX-R1 immunoreactivity as for controls, and was also devoid of OX-R2 immunoreactivity. Ganglion cells, amacrine cells, and cell processes within the inner and outer plexiform layer were positive for all three antigens. The only difference was the obvious decrease in staining

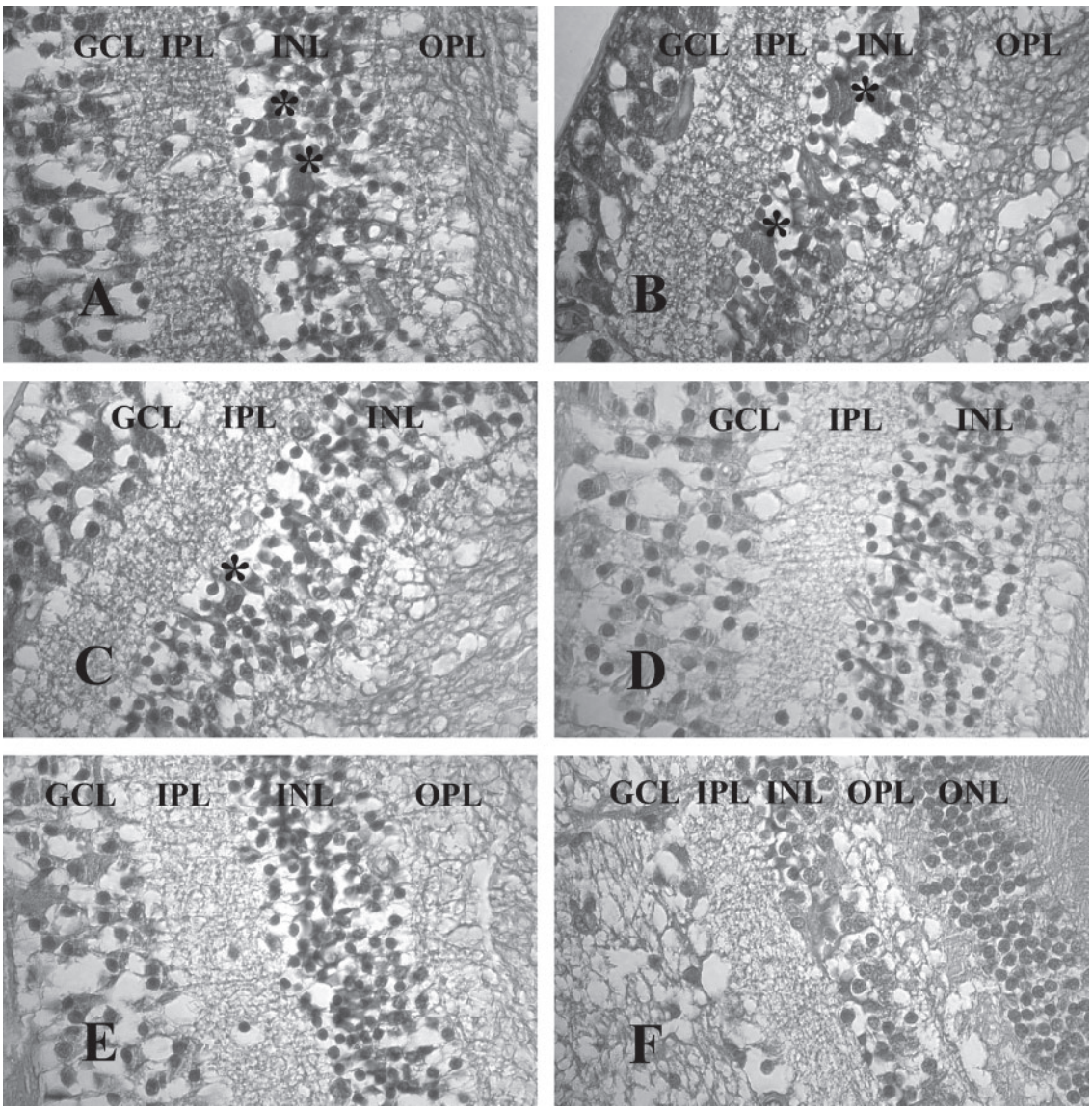

Fig. 1. Orexin-A-, orexin-B- and OX-R1-immunoreactive structures (red deposits) in the retina. GCL = Ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer. Ganglion cells in the GCL, amacrine cells (asterisks) in the INL, and cell processes in the IPL and OPL are immunoreactive for orexin A (**A**), orexin B (**B**) and OX-R1 in controls (**C**). **D** OX-R2 immunoreactivity is missing in a simultaneously stained section. **E** Control section stained simultaneously according to the same procedure, with the exception that the primary antibody was omitted. The lack of immunoreactivity confirms the specificity of the reaction. **F** The overall intensity of orexin A immunoreactivity is decreased in the retina of an AD patient. **A–F** Original magnification ×242.

intensity for all observed antigens (table 2). In contrast to the control cases, overall OX-R1 immunoreactivity was weak in both AD cases with decreased orexin A or orexin B staining.

Discussion

The present results provide the first immunohistochemical description of orexin A, orexin B and OX-R1 distribution in human retina. Neuronal retinal cells and their cellular processes were the main localization sites,

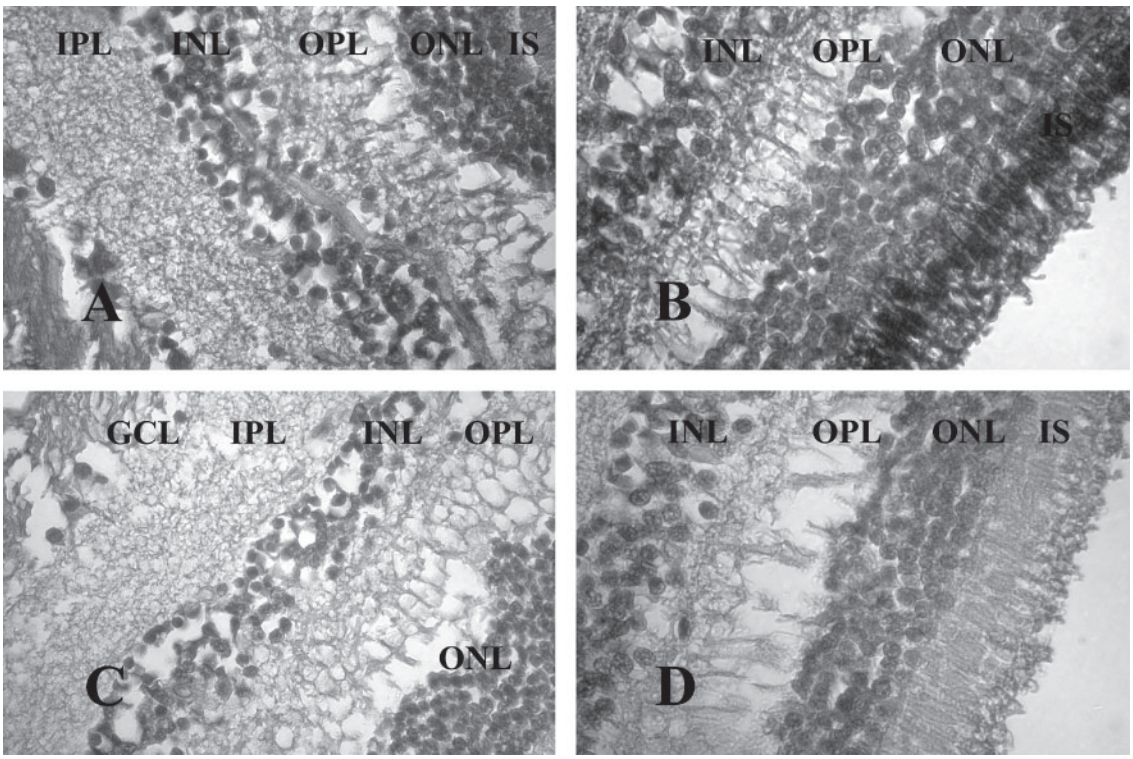

Fig. 2. Orexin-A- (**A**) and OX-R1- (**B**) immunoreactive structures in outer layers of the retina including the positively stained inner segments (IS) of the photoreceptor cells adjacent to the outer nuclear layer (ONL). **C**, **D** Control sections. **A–D** Original magnification ×242.

with ganglion and amacrine cells being most prominent. OX-R2 immunoreactivity was entirely absent. OX-R1 may be the main receptor transducing the effects of both orexin A and orexin B in the retina. An alternative explanation for the absence of OX-R2 immunoreactivity may be that the subjects were elderly, since brain OX-R2 mRNA levels show an age-related decline [28].

Certain retinal ganglion cells appear to be intrinsically photosensitive [29] and transmit photic information to the SCN, the site of the master circadian clock, via a direct retinohypothalamic tract [22, 23]. Therefore, these cells play an important role in light entrainment of circadian rhythms. Our data mark ganglion and amacrine cells as the main localization site of orexin A, orexin B and OX-R1 suggesting a role for orexins as regulatory peptides for the retinohypothalamic tract.

In rats, orexin A has been found to modulate the sleep-wake cycle [30]. Intracerebroventricularly administered orexin A enhanced arousal. This effect can be traced back to the dense innervation of orexinergic nerves in the locus ceruleus [30]. In the retina, orexins may act on ganglion and amacrine cells as participants of the circadian system. These cells are also susceptible to the regulatory action of an important circadian modulatory hormone, melatonin [24, 31]. Dopaminergic and GABAergic output of the amacrine cells is directly regulated by melatonin [31]. Since orexin-A- and orexin-B-positive fibers [9] and OX-R1 expression [16] have been localized in the SCN, orexins may be involved in the retinohypothalamic tract from the amacrine and ganglion cells projecting to the SCN. Indeed, the distribution of orexin fibers and the orexin-induced c-fos expression in the SCN suggest that orexins may activate many neurons of the circadian pacemaker directly [9] and thus be involved in circadian regulation [17].

The present data on 2 AD cases are too preliminary to allow disease-specific interpretations. However, the reduced overall intensity of all observed antigens is of interest. AD pathology is complex, involving different neurotransmitter systems and resulting in cognitive, circadian and behavioral disturbances [26], as well as eating disorders [32], which partly overlap with orexin-related effects. Orexins may contribute via entrainment pathways, sleep and feeding behavior to AD pathology and clinical symptomatology.

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